

# **Why is Asp-286 Conserved in the V-ATPase?**

An Honors Thesis (HONRS 499)

by

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A handwritten signature in dark ink, reading "Karlett Parra-Belky", written over a horizontal line.

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May 2005

Graduation Date: May 2005

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**NARRATIVE:**

V-ATPases are complex proteins found in most cells that are made up of many components, or subunits, organized into two domains called  $V_1$  and  $V_0$ . One of the subunits, called subunit d, is interesting because it holds the two domains of the protein together. In order to determine the role of subunit d in the V-ATPase, an amino acid of the subunit was changed by mutagenesis to see how the protein function was altered.

This research is important because the V-ATPase is essential for several physiological processes within the cell. It is responsible for the acidification of many intracellular compartments. Maintaining a specific acidity is crucial for many physiological activities to occur such as bone resorption in osteoclasts, neurotransmitter sequestration in secretory granules, and sperm development in vas deferens. Many health problems such as cancer, osteoporosis and kidney failure have been linked to V-ATPases. By determining the role of subunit d in V-ATPase function, the complex as a whole will be better understood. In the long-term this project will provide the foundation to develop drugs that will help prevent health problems involving V-ATPases.

**ACKNOWLEDGEMENTS:**

- I want to thank Dr. Karlett Parra-Belky for giving me the opportunity to work on such an interesting research project and for being such an excellent mentor.
- I would also like to thank Dr. Donald Pappas for his guidance and support in the laboratory.

## **ABSTRACT:**

V-ATPases are multi-subunit proton pumps found on the cellular membranes of most organisms from yeast (*Saccharomyces cerevisiae*) to humans. They are composed of two main components capable of reversible disassembly:  $V_1$  and  $V_o$ .  $V_1$  is composed of eight different subunits and is the peripheral domain, whereas  $V_o$  is composed of five different subunits and is the integral domain. The long-term objective of this research is to determine the function of one of these subunits on the  $V_o$  complex: subunit d, encoded by the VMA6 gene. Subunit d is of interest because it serves as the bridge between  $V_1$  and  $V_o$ .

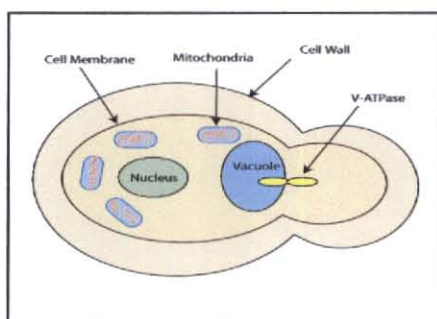
Asp-286 of subunit d is highly conserved in mammals, insects and fungi, suggesting that it may be important for V-ATPase function. Using QuikChange site-directed mutagenesis by PCR, a mutation was introduced in subunit d. The mutation substituted aspartic acid on position 286 with alanine. We hypothesized that if Asp-286 has a structural role in subunit d, this change would modify the tertiary structure of subunit d and possibly alter the activity and assembly of the V-ATPase complex.

## Why is Asp-286 Conserved in the V-ATPase?

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### INTRODUCTION:

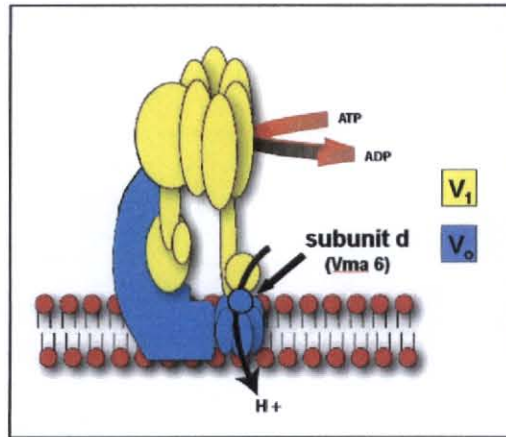
V-ATPases are ATP-driven multi-subunit proton pumps found on the vacuolar membrane (see Figure-1) of yeast (*Saccharomyces cerevisiae*) and the endomembrane systems of other organisms including humans. V-ATPases are associated with the acidification of intracellular compartments such as Golgi apparatus, endosomes, vacuoles, lysosomes, and clathrin-coated vesicles. Maintaining a specific acidity is crucial for many physiological activities to occur such as receptor-mediated endocytosis, bone resorption in osteoclasts, neurotransmitter sequestration in secretory granules, and sperm development in vas deferens. Many health problems such as cancer, osteoporosis and kidney failure have been linked to V-ATPases. In the long-term, this project will provide the foundation to develop drugs that will help prevent health problems involving V-ATPases by determining the role of subunit d for V-ATPase function.



**Figure-1:** V-ATPases are present on the vacuolar membrane in yeast.

V-ATPases are composed of two main components capable of reversible disassembly,  $V_1$  and  $V_o$ .  $V_1$  is composed of eight different subunits and is peripherally attached to the membrane, whereas  $V_o$  is composed of five different subunits and is the integral membrane domain. During periods of glucose starvation, the complex is disassembled into  $V_1$  and  $V_o$ , but when glucose is abundant, the complexes are assembled and active. The long-term objective of this research is to

determine the function of one of these subunits on the  $V_o$  complex: subunit d, encoded by the VMA6 gene (see Figure-2). Subunit d is of interest because it serves as the bridge between  $V_1$  and  $V_o$ .



**Figure-2:** Subunit d is part of the  $V_o$  domain in the V-ATPase complex.

Specifically, this research project was aimed to make a mutation on subunit d of the V-ATPase in the yeast *Saccharomyces cerevisiae*, in order to determine how the mutation affected the biological function of the V-ATPase complex.

## HYPOTHESIS:

Aspartic 286 (Asp-286) is highly conserved in the V-ATPase subunit d of mammals, insects and fungi, suggesting that it may be important for V-ATPase function. Using QuikChange site-directed mutagenesis by PCR, a new strand of DNA containing a mutation in subunit d was introduced into yeast. The mutation substituted aspartic acid on position 286 with alanine. It was anticipated that if Asp-286 was important for V-ATPase function, its mutation could modify the structure of the protein, it could alter assembly of the V-ATPase complex, and/or it could affect the enzyme's biological functions.



## METHODS:

*V-ATPase Subunit d Sequence Alignment:* The sequence of V-ATPase subunit d from five different species (frog, cow, house mouse, human and yeast) were aligned using the Clustalw program online.

*Mutagenic Primer Design:* Using various databases (see references), the DNA sequence for the gene encoding subunit d was found. The codon corresponding to the amino acid aspartic acid 286 was identified and two primers (44 bases length) were designed with melting temperatures greater than 78°C. The QuikChange Site-Directed Mutagenesis Kit from Stratagene was used to introduce the mutation.

*Quikchange Site-Directed Mutagenesis:* Reaction mixtures were prepared containing reaction buffer, dsDNA template, the mutagenic oligonucleotide primer and its complementary oligonucleotide primer, dNTP mix, ddH<sub>2</sub>O, and PfuTurbo DNA polymerase in a final volume of 50 µl. The reaction was cycled 16 times and placed on ice for 2 minutes before the methylase enzyme, DpnI, was added to each reaction. Samples were mixed, spun down, and incubated at 37°C for 1 hour. Reactions were stored at -20°C.

*Transformation of competent E.coli Cells:* Dpn I-treated DNA from the PCR reaction was mixed with competent cells. Cells plus mutagenized plasmid DNA were incubated on ice for 30 minutes. The reaction was heat pulsed for 45 seconds at 42°C and then placed on ice for 2 minutes. 0.5 ml of preheated NZY broth was added and the cells recovered at 37°C for 1 hour with shaking at 225-250 rpm. Two aliquots of 250 µl each containing transformed cells were plated on agar plates (LB and AMP) and allowed to grow for approximately 20 hours at 37°C.

*Miniprep:* BioRad Quantum Minipreps were performed following the manufacturer's protocol. Briefly, cells were transferred to a microcentrifuge tube and pelleted by centrifugation for 30 seconds. The supernatant was aspirated and Cell Resuspension Solution (Buffer pH 7) was added. Cells were vortexed until resuspended and Cell Lysis Solution (NaOH plus SDS) added and mixed by inversion approximately 10 times. After adding Neutralization solution (Potassium Acetate), the cell debris was pelleted by centrifugation for 5 minutes in a microfuge. A Spin Filter was inserted into one of 2 ml wash tubes and the lysate (supernatant from centrifuging) transferred into the Spin Filter. 200  $\mu$ l of matrix was added and after centrifugation for 30 seconds, the Spin Filter was washed with 500  $\mu$ l of Wash Buffer (containing Ethanol) by centrifugation for 2 minutes. The filter was placed in a tube and 50  $\mu$ l of deionized water pre-warmed at 70°C was added to elute the DNA which was stored in the freezer at -20°C. The purified DNA was sequenced at the Iowa State University Sequencing Facility and the mutation confirmed.

*Yeast Transformation:* A cell culture was grown to mid-log phase in YEPD pH 5 and cells (4-5 ml) harvested at 3000 rpm for 5 min. Pellets were washed with 1 mL deionized water and then with 1 ml TE/LiAc. After washes, the pellets were resuspended in approximately 50  $\mu$ l of TE/LiAc and transformation mixtures made by mixing 5  $\mu$ l testis DNA, 2  $\mu$ l plasmid DNA, 50  $\mu$ l cells, and 300  $\mu$ l 40% PEG/TE/LiAc. Mixtures were vortexed and incubated at 30°C for 30 min. Cells were heat-shocked at 42°C for 10 min. After incubation, 1 ml of deionized water was added, cells centrifuged for 2 min and the supernatant aspirated. Cells were resuspended in 100  $\mu$ l deionized water and plated onto SD-Ura pH 5 media.

After the yeast transformations were completed, the effect of the mutation D286A on the cell growth characteristics and the V-ATPase complex function was studied. A comparative



analysis of a wild-type strain and the mutant strain was performed by doing serial dilution spotting, ATPase assays and Western blots analyses of whole lysates and vacuolar vesicles.

*Serial Dilution Spotting:* Wild-type and D286A cell cultures were grown to mid-log phase in SD-Ura pH 5. Equal amounts of cells from each culture were harvested and centrifuged for 5 min. The cells were resuspended in 200  $\mu$ l ddH<sub>2</sub>O. This concentrate was used to make dilutions of  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$  and  $10^{-4}$  cells. The sample dilutions were spotted onto 6 plates (two SD-Ura pH 5, two SD-Ura pH 7.5, and two SD-Ura pH 7.5 + CaCl<sub>2</sub>) in drops of 5  $\mu$ l. One set of plates was incubated at 30°C and other at 37°C for 72 hours.

*Whole Cell Lysis:* Cell cultures were grown to mid-log phase in SD-Ura pH 5 and were harvested in a clinical centrifuge at position #5 for 3 min. After decanting the supernatant, pellets were resuspended in 3 ml of 0.1 M Tris-HCl pH 9.4 + 10 mM DTT and rocked for 5 min at 30°C. The samples were centrifuged for 3 min at position #5 and the supernatant aspirated. Cells were washed by resuspending with 13 ml 10mM Tris-HCl pH 7.5 + 1.2 M Sorbitol + 2% Glucose (Solution A), centrifuging for 3 min at position #5 and decanting the supernatant. Pellets were resuspended in 3 ml of Solution A and 2  $\mu$ l Zymolase (10 $\mu$ g/ $\mu$ l) was added to digest cell walls. After incubating the samples at 30°C for 20 min, they were centrifuged for 5 min at position #3 and washed three times with 13 ml Solution A. Samples were washed one additional time, resuspending in 1 ml Solution A. Samples were transferred to a microfuge tube and centrifuged for 1 min. After the supernatant was aspirated, the pellets were resuspended in 100  $\mu$ l cracking buffer containing 5%  $\beta$ -ME (pre-warmed to 50°C) and the samples were heated in a heating block at 50°C for 20 min, vortexing periodically until the pellet was dissolved. Samples were centrifuged for 1 min and stored in the freezer at -20°C.

*Vacuolar Preparation:* Cell cultures were grown to mid-log phase in 1 L SD-Ura pH 5 and the cells were harvested at 5000 rpm for 5 min. After decanting the supernatant, cells were washed twice with 2% glucose. Cells were resuspended in 100 ml of 1.2 M sorbitol + 2% glucose + 10 mM Tris-HCl pH 7.5 per liter of culture. The pH was adjusted to 7.0 using Tris-HCl pH 7.5 and 400 units of zymolase were added per 4000 OD of cells at 600 nm. The cells were placed in a shaker at 150 rpm and 30°C for 60-90 min until they were converted to spheroplasts. Spheroplasts were collected by centrifugation at 3500 rpm for 5 min and washed twice with 1.2 M sorbitol + YEPD (yeast extract, peptone, dextrose). Then 20 ml Buffer A (10 mM MES-Tris pH 6.9 + 0.1 mM  $MgCl_2$  + 12% Ficoll 400) containing protease inhibitors (PMSF, Leupeptin, Pepstatin, Aprotinin and Chymostatin) was added to the spheroplasts for every liter of original culture. The spheroplasts were homogenized on ice in a pre-chilled Dounce homogenizer for 5 min and the lysate was transferred to pre-chilled polyallomer tubes. Buffer A was layered on top of the lysate in each tube and each sample was centrifuged in the ultracentrifuge (pre-chilled to 4°C) at 24,000 rpm for 35 min. The wafers were collected from the top of the centrifuge tubes and added to 2-3 ml 15 mM MES-Tris pH 7.0 + 5% glycerol (on ice) per liter of original culture. The purified vacuolar vesicles were homogenized by pipetting several times and 250  $\mu$ l samples were aliquoted into microfuge tubes. Samples were stored in the freezer at -80°C.

*ATPase Assays:* Assay mixtures (containing KCl, Tris base,  $MgCl_2$ , NADH, PEP (phosphoenol pyruvate), ATP, L-lactic dehydrogenase and pyruvate kinase) were thawed and warmed to 30°C. The UV-Visible spectrophotometer was set-up for a time-based collection of 300 sec total (1 reading per second) at 340 nm at 37°C. Less than 100  $\mu$ l of vacuolar vesicles were mixed into the cuvette containing the assay mixture (1 ml) and the data collection was initiated immediately. After the data collection ended, the sample in the cuvette was mixed and the measurement was repeated for an additional 300 sec twice.

*Western Blots:* The 10% SDS-PAGE (separating) gel was prepared by combining deionized water, acrylamide/bis, Tris-HCl pH 8.8 and SDS in a side-armed flask and degassed by using a vacuum trap for 10-15 min. After adding APS and TEMED, the mixture was poured into the gel apparatus and allowed to polymerize for 30 min. The 4% SDS-PAGE (stacking) gel was prepared by combining ddH<sub>2</sub>O, acrylamide/bis, Tris-HCl pH 6.8 and SDS. After degassing for 10-15 min using a vacuum trap, APS and TEMED were added and the mixture was poured into the gel apparatus over the polymerized separating gel and allowed to polymerize for 30 min. The gel was loaded into the electrophoresis equipment and the chamber was filled with 1 X Running Buffer. After loading the appropriate volumes of the standard marker and each sample, the chamber was plugged into a power supply and set at 150 V until the samples migrated to the end of the gel. The electrophoresis was turned off and the gel was removed from the glass plates and positioned with a nitrocellulose membrane between two pieces of Whatman paper in 1 X Transfer Buffer. This sandwich was placed inside a transfer cassette and loaded into the Transblot apparatus, which was set-up to run overnight first at 150 mA and then for at least 2 hours at 200 mA. The membrane was removed and rinsed first with 1 X TTBS (composed of Tris pH 7.5, NaCl, Tween 20 and Millipore water) and then with a Blotto solution. The membrane was incubated on the rocker for a minimum of 4 hours in the primary antibody (Top half: 1°  $\alpha$ -60, 69, 100, Bottom half: 1°  $\alpha$ -27, 30, 36) at room temperature. The primary antibody was removed and the membrane was rinsed with 1 X TTBS. The membrane was incubated in alkaline phosphatase-conjugated (AP) secondary antibody (Top half: 2°  $\alpha$ -Mouse, Bottom half: 2°  $\alpha$ -Rabbit) for a minimum of one hour. After incubation, the antibody was removed and the membrane was rinsed in 1 X TTBS for 5 min. Fresh developing solution (composed of the AP substrates, NBT and BCIP solution) was poured over the membrane and the blot was allowed to develop for five minutes. The membrane was rinsed with Millipore water after the blot was fully developed.



**RESULTS:**

A portion of the sequencing alignment of the VMA6 gene of 5 species is shown in Table-1. This showed that aspartic acid (D) is highly conserved as amino acid 286th in the subunit d from yeast to human.

FROG	L	E	D	R	F	F	E
COW	L	E	D <sub>214</sub>	R	F	F	E
HOUSEMOUSE	L	E	D	R	F	F	E
HUMAN	L	E	D <sub>292</sub>	R	F	F	E
YEAST	L	E	D <sub>286</sub>	H	F	Y	Q
	*	*	*	:	*	:	:

**Table-1:** Sequencing alignment of subunit d across species shows that D is highly conserved as amino acid 286<sup>th</sup>.

Because D286 is highly conserved, it was identified as a good candidate for our mutagenesis analyses. The mutation made changed the residue to alanine (D286A).

Each subunit of the V-ATPase is a protein encoded by a specific codon (triplet) within the genetic code. The genetic code is made up of nucleic acid sequences that are translated into amino acid sequences in the cell. Using bioinformatics a small DNA fragment, or oligonucleotide, was designed. This oligonucleotide was used as a primer in DNA synthesis and it was responsible for changing one codon (triplet) in the sequence of the gene VMA6 to create a mutant strand of DNA. The procedure of replacing one amino acid with another in the mutation is called site-directed mutagenesis.

The sequence of the primer designed contained one nucleotide changed, in which the codon corresponding to the aspartic acid (GAT) was changed to alanine (GCT). The sequence for the two primers used is shown on the following page.

<i>D286A (1)</i>	GAT→ GCT
5' GAGACTGGTAACTTAGAA <u>GCT</u> CACCTTTACCAATTGGAAATGG	
3' CCTCTGACCATTGAATCT <u>TTCG</u> AGTGAAAATGGTTAACCTTTACC	
<i>D286A (2) comp.</i>	
5' CCATTTCCAATTGGTAAAAGTGAGCTTCTAAGTTACCAGTCTCC	

The percent of GC (%GC) in the primers was determined by dividing the number of GC that were conserved by the primer's length in base pairs:

$$\%GC = 17/44 = \mathbf{41\%}$$

The percent of mismatched nucleotides was determined by dividing the number of nucleotides changed by the primer's length in base pairs:

$$\% \text{ mismatch} = 1/44 = 0.0227 = \mathbf{2\%}$$

The melting temperature ( $T_m$ ) was determined by the following calculation:

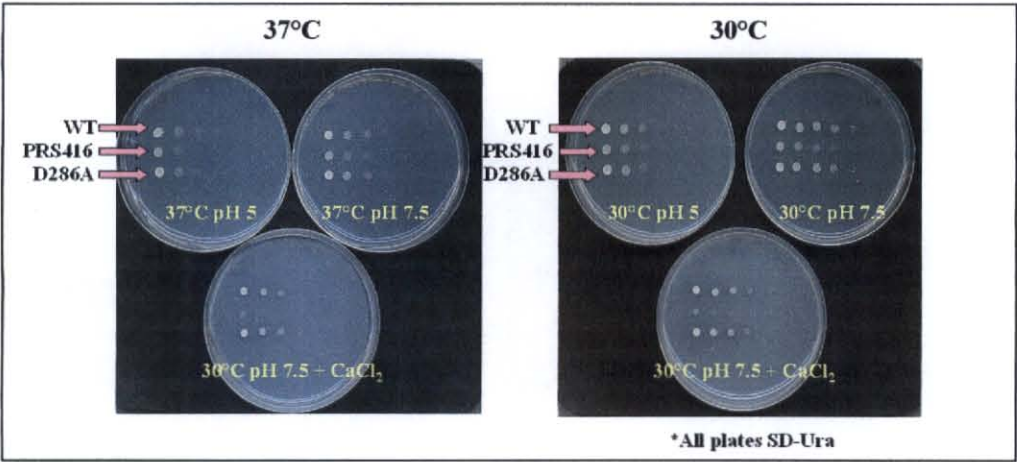
$$T_m = 81.5 + 0.41(\%GC) - 675/N - \% \text{ mismatch} = 80.96^\circ\text{C} = \mathbf{81\%}$$

The mutation was made using Quikchange site-directed mutagenesis and the reactions used to transform competent *E. coli* cells. Following, the DNA was extracted and purified from the transformed *E. coli* cells and the mutation confirmed by DNA sequencing. The yeast *vma6Δ* strain was transformed with the mutagenized gene cloned in the plasmid pRS316 and experiments were performed to compare the cells expressing the mutant subunit d (*vma6*-D286A) and *vma6Δ* cells expressing the wild-type gene in the same plasmid.

Serial dilution spotting analyses were performed to examine the effect of the mutation on the cell growth phenotype. Results are shown in Figure-3. Cells were plated on SD-Ura adjusted to pH 5, pH 7.5 and pH 7.5 plus  $\text{CaCl}_2$  and each set of plates was grown at 30°C and 37°C for 48 hours. The cells shown in Figure-3, (left) were grown at 37°C and the cells shown on the right were grown at 30°C. Cells expressing the mutant subunit d (*vma6*-D286A) exhibited wild-type

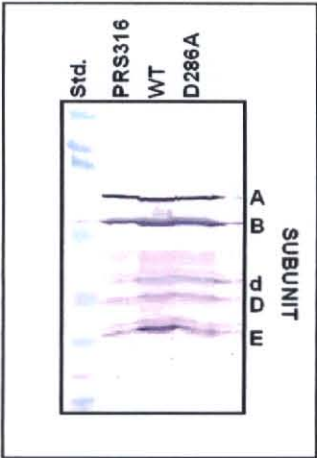


growth at pH 5, pH 7.5 and with CaCl<sub>2</sub>. In addition, the mutant strain did not show temperature sensitivity because it grew equally to the wild-type strain at 30°C and 37°C.



**Figure-3:** Serial dilution spotting results indicated that the mutant strain did not show *vma* phenotype or temperature sensitivity.

A Western blot of the whole cell lysis is shown in Figure-4. All the subunits of the V-ATPase complex, represented by a band on the gel, were shown below. The samples used were the pRS316 vector (negative control), wild-type (VMA6-pRS316) and the D286A mutant cells. The wild-type strain served as the positive control. All of the protein bands present in the wild-type strain (V-ATPase subunits A, B, d, D and E) are also present in the D286A mutant strain at similar levels.



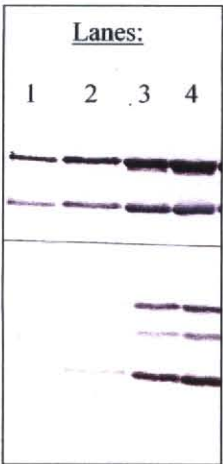
**Figure-4:** Western blot of whole cell lysis showed that all of the subunits of the V-ATPase complex were present in the D286A mutant at levels comparable to the wild-type cells, indicating that there was not subunit degradation due to the mutation.

Density gradient centrifugation was used to isolate the vacuolar membrane from the yeast cells. Vacuoles contain V-ATPase complexes assembled that are responsible for maintaining the vacuolar acidic pH. Using Lowry assays to determine the concentration of protein present in the membrane and ATPase assays to measure Concanamycin-sensitive ATPase activity, we calculate the specific activity of the V-ATPase at the membranes. The assay results are summarized in Table-2.

Cell Strain	Protein Conc. (µg/µL)	Concanamycin-sensitive ATPase Specific Activity (µmol Pi /min /mg)
WT	0.25	0.436
D286A	0.32	0.711

**Table-2:** Data from ATPase assays indicated that D286A mutant vacuolar membranes were 38% more active than the wild-type vacuolar membranes.

In order to visualize and compare the amount of V-ATPase subunits present at the vacuolar membranes, serial dilutions of wild-type and mutant vesicles were subjected to SDS-PAGE followed by Western blot analysis using antibodies against the V-ATPase subunits A, B, d, D and E. The results are shown in Figure-5. The amount of protein loaded per well is indicated in Table-3. The western blots show that the wild-type and mutant vacuolar membranes contain the same amount of vacuolar protein.



LANE	CELL STRAIN	µg VESICLES
1	WT	2
2	D286A	2
3	WT	10
4	D286A	10

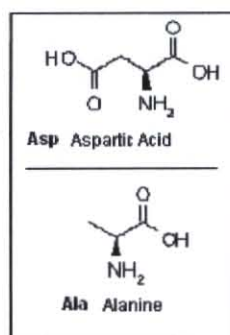
**Table-3:** Volume of vacuolar protein loaded per well.

**Figure-5:** Vacuolar vesicle gel Western blot results showed no difference detected in the amount of vacuolar protein present in the mutant and wild-type membranes.

## DISCUSSION:

The place where to make an appropriate and effective mutation was determined by identifying the amino acids conserved in subunit d. This information was obtained by aligning and comparing the amino acid sequence of subunit d among several species. Because the functionally most important residues of a protein are fully conserved, including D286 of subunit d, these residues are good candidates for mutagenesis experiments so they are represented by an asterisk in Table-1. In this study, we mutated the VMA6 gene. Cloned in the pDNA (pRS316), VMA6 encodes for subunit d of the V-ATPase and the mutation made was D286A, “D” represents the amino acid being changed (aspartic acid), the numeric identification “286” indicates the location of the amino acid in the sequence of subunit d and “A” is the replacement amino acid (the mutation: alanine.)

We replaced aspartic acid with alanine because their structures are different in size (see Figure-6), and the chemical properties of the two amino acids are different too. Aspartic acid is polar and negatively charged at a physiological pH while alanine, however, is a non-polar amino acid of smaller size. We anticipated that these differences in size and charge could potentially alter the tertiary structure of the protein and even more, the assembly of  $V_1V_o$  complexes. It was possible that any electronegative interactions, once involving D286, may no longer occur without the carboxylate group and side chains in subunit d may interact with the newly added alanine in a different way. We hypothesize that such a small change could, therefore, potentially change the resulting tertiary structure of the protein. This would affect how the subunit interacts with other subunits within the V-ATPase and, ultimately, how the proton pump complex functions and interacts as a whole.



**Figure-6:** Aspartic acid was replaced by alanine because their structures differ in size and chemical properties.

By using polymerase chain reaction (PCR), the template DNA was mutagenized and amplified. Transformation of *E. coli* was accomplished by heat shocking XL-1-Blue competent cells in the presence of the mutagenized pDNA. The heat shock caused the formation of transient pores on the membrane, allowing the DNA bound to enter in to the cell and transforming them. The cells that received the pDNA (transformed cells) were separated from those that were not transformed by including ampicillin in the growth medium. The transformed cells were able to survive because they carried an ampicillin-resistant gene in the pDNA. Colonies were selected and miniprep performed and further examined by sequencing which confirmed the presence of the desired mutation within the gene VMA6.

The mutant DNA was used to transform yeast by first treating the *vma6Δ* cells with PEG and then adding TE/LiAc to aid DNA in entering the cell. The cells were heat shocked, which opened up membranous pores and allowed the new DNA to enter the cell. The transformed cells were then plated using SD-Ura pH 5 media. Serial dilutions of cells transformed with the wild-type gene, the vector and the mutant *vma6-D286A* were examined to establish whether the mutant shows *vma* phenotype, which is a conditionally lethal phenotype in which cells grow at pH 5 but fail to grow at pH 7.5 and in the presence of  $\text{CaCl}_2$ . This phenotype occurs when the activity of mutant V-ATPases is less than 20% of the wild-type's V-ATPase activity. Serial



dilutions (shown in Figure-3) indicated that the mutation D286A does not show *vma* phenotype; it shows wild-type growth at neutral pH and with  $\text{CaCl}_2$  and therefore maintains at least 20% activity. The mutant was not temperature sensitive, because it grew well at both temperatures.

To further study this mutant, a whole cell lysis was performed on wild-type cells, D286A mutant cells and cells expressing the pRS316 vector alone. Proteins in the whole cell lysate were then separated by SDS-PAGE and Western blots were carried out to visualize the stability and expression of the V-ATPase components in each sample (shown in Figure-4) by using antibodies against V-ATPase subunits. Absence of a subunit in the mutant cell would have indicated that the subunit was degraded as a result of the mutation or that the subunit was not made in the cell. Results showed that all of the subunits of the V-ATPase complex were present in the D286A mutant at levels comparable to the wild-type cells, indicating that there was not subunit degradation due to the mutation.

Vacuolar preparations of the wild-type and D286A mutant cells were used to study the complex. Because purified vacuolar vesicles contain V-ATPase complexes, they were used to examine ATPase activity and assembly of  $V_1$  and  $V_o$ . The wild-type membranes had a protein concentration of  $0.25 \mu\text{g}/\mu\text{L}$  and the D286A mutant membranes a concentration of  $0.32 \mu\text{g}/\mu\text{L}$  (see Table-2). Next, an enzymatic coupled ATPase assay was used to measure ATP hydrolysis at the membranes. The UV-Visible spectrophotometer was used to measure the oxidation of NADH to  $\text{NAD}^+$  at 340 nm. This reaction was coupled with the hydrolysis of ATP to ADP by the V-ATPase complex. By coupling the reactions of three enzymes (V-ATPase, pyruvate kinase and lactate dehydrogenase), ATP was regenerated and there was an unlimited supply of ATP to the V-ATPase. For every one mole of ATP that the V-ATPase hydrolyzed, 1 mole of NADH was oxidized. Because absorbance was measured at a wavelength of 340 nm, which is the maximum absorbance of NADH, the assay curve provided the rate of ATP hydrolysis. Each sample was

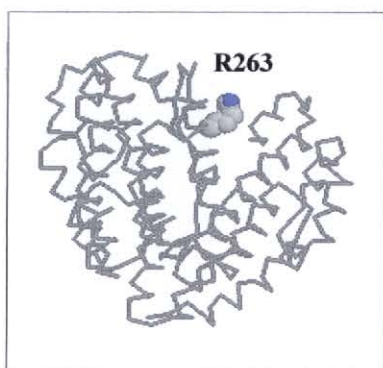


assayed twice; during the first ATPase assay the vesicles were added alone to the mixture in the cuvette in order to determine the total ATPase activity on the membranes. For the second assay, the vesicles were mixed with concanamycin A, a vacuolar ATPase specific inhibitor, which allowed us to determine the concanamycin-sensitive ATPase activity (V-ATPase activity). Using this information as well as the protein measured, the specific activities of the wild-type and mutant D286A membranes were found. The specific activity of the wild-type and mutant membranes was determined to be 0.436 units/mg and 0.711 units/mg, respectively, where units are  $\mu\text{mol P}_i/\text{min}$ . Our results indicated that D286A mutant vacuolar membranes are 38% more active than the wild-type vacuolar membranes. This observation could be explained if the mutant membranes contained more  $V_1V_o$  assembled than the wild-type; in other words, if mutant membranes have more V-ATPase complexes present.

In order to determine the amount of V-ATPase subunits present on the wild-type and mutant membranes, a gel was prepared containing vacuolar vesicles. Vacuolar protein was separated using SDS-PAGE and by loading increasing amounts of protein (see Table-3) (0.5  $\mu\text{g}$ , 1  $\mu\text{g}$ , 2  $\mu\text{g}$ , 10  $\mu\text{g}$  and 20  $\mu\text{g}$ ) although only 2  $\mu\text{g}$  vs. 10  $\mu\text{g}$  are shown in Figure-5. Quantitative immunoblots served to compare the level of  $V_1$  and  $V_o$  subunits in D286A and wild-type membranes. The results, which are shown in Figure-5, indicated no difference detected in the amount of vacuolar protein present in the mutant and wild-type membranes. Therefore, our results indicated that D286A mutant membranes do not have more V-ATPase complexes than wild-type vacuolar membranes, suggesting that the mutant V-ATPase displays greater activity than the wild-type membrane.

X-ray crystallography structures of the V-ATPase subunit d in yeast have not yet been obtained. However, the V-ATPase subunit d of *T. thermophilus* (bacteria) is available and is shown in Figure-7. Using this image and the RasMol program, the site of the mutation on subunit

d can be visualized and it could provide important information about the interactions between D286 and other residues. The sequencing alignment shows that Asp-286 (D286) in yeast is conserved as Arg-263 (R263) in *T. thermophilus*. This is a conservative substitution because both aspartic acid and arginine (Arg) are charged amino acids. Aspartic acid is negatively-charged and acidic while arginine is positively-charged and basic at physiological pH. Figure-7 shows the location of R263 in subunit d which is very interesting. The conserved amino acid is on the surface close to the region predicted to bind to the  $V_1$  domain. This suggests that this particular site may be important for coupling  $V_1$  and  $V_o$  structurally and functionally. If an effect of the mutation on the central stalk that connects  $V_1$  and  $V_o$  (rotor) could be transmitted, by conformational changes, to the ATP hydrolytic sites, it could have contributed to the enhanced activity detected *in vitro*.



**Figure-7:** Subunit d in *T. thermophilus* structure obtained using RasMol and location of R263.

## FUTURE DIRECTIONS:

Comparative studies of D286A mutant cells with the wild-type strain will be continued and these results must be reproduced. Additional vacuolar preparations and assays will be performed to confirm the data herein presented and the information collected will be used to better understand the functional role of subunit d in V-ATPase.

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